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**In vivo high-frequency ultrasound for the characterization of thrombi
associated with aortic aneurysms in an experimental mouse model**

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Jansen, High-frequency ultrasound for the assessment of AAA associated thrombi

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Abstract

The development of abdominal aortic aneurysm (AAA) associated thrombi play an important role during the onset and progression of AAAs. The aim of this study was to evaluate the potential of high-frequency-ultrasound for characterization of AAA associated thrombi in an apolipoprotein-E-deficient (ApoE^{-/-}) mouse-model. Ultrasound measurements were performed using a high-resolution ultrasound-system (Vevo770, VisualSonics, Canada) with a 30MHz linear-array-transducer (RMV707B). Magnetic resonance imaging with a 3 Tesla scanner (Achieva MR system, PhilipsHealthcare, Best, Netherlands) and a single-loop microscopy-coil was performed as a reference standard.

All stages of aneurysm development were evaluated by histological analyses. The “signal-thrombus-matrix” to “signal-blood” ratio on high-frequency ultrasound-measurements showed a strong correlation ($R^2=0.81, p<0.05$) with the state of extracellular-matrix-remodeling. Furthermore, size measurements derived from the high-frequency-ultrasound correlated well with MRI and histology.

This study demonstrated, that high-frequency-ultrasound enables the reliable in vivo quantification of extracellular matrix remodeling at different stages of thrombus development based on the thrombus echogenicity.

53 **Key Words:** Abdominal aortic aneurysms; ultrasound; magnetic resonance imaging

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Introduction

Cardiovascular diseases are consistently ranked among the leading causes of death in developed and developing countries (Krishnan, et al. 2010). Among cardiovascular diseases, abdominal aortic aneurysms (AAAs) represent the third leading cause of death. As a result of the increased aging of the population, their incidence is constantly rising (Hallett 2000). If undiagnosed or untreated, AAAs can increase in size over time, which is associated with a higher risk of rupture (Thompson, et al. 2002).

It is widely assumed that the rupture of the elastic laminae in the tunica media leads to a dissection of the aortic wall, which subsequently results in the development of an aortic aneurysm. Simultaneously, the blood clotting cascade is activated, causing the formation of a small focal fibrin-rich hematoma at the rupture site (Saraff, et al. 2003). If the aneurysm continues to increase in size, a large fibrin-rich thrombus develops. During the early stage of AAA development, the thrombus is primarily composed of fibrin, which is associated with the coagulation of blood in the aneurysmatic vessel wall. During the further development of the aneurysm, the fibrin rich thrombus is increasingly stabilized due to extracellular matrix expression by macrophages and smooth muscle cells. This leads to the development of an extracellular matrix rich in elastin and collagen, while the hemorrhage associated fibrin is subsequently replaced

During further progression of the aortic aneurysm, there are different multifactorial pathways, resulting either in a further extracellular matrix synthesis and stabilization of the thrombus matrix or to a degradation of the extracellular matrix with a potential destabilization of the thrombus. This process is thought to be highly relevant for the outcome of AAAs (Huffman, et al. 2000, Krettek, et al. 2003, Saraff, et al. 2003). Therefore, the detection and characterization of AAA associated thrombi might represent an important factor for the further evaluation of aneurysms.

Even though human AAAs represent a relatively frequent vascular disease, their pathogenesis is not fully understood yet. This is because of the difficulty to obtain in vivo tissue samples from aortic aneurysms. Therefore, pre-clinical animal models of AAAs are important to increase our understanding of the different factors which cause the development and destabilization of aortic aneurysms. For the generation of AAAs several different small animal models of AAA have been described (Trollope, et al. 2011). The administration of Angiotensin II (AngII) via subcutaneous implanted osmotic minipumps in apolipoprotein E-deficient (ApoE^{-/-}) mice represents the most frequently used technique, as AAAs develop spontaneously without requiring surgical intervention at the aorta (Daugherty, et al. 2000). Previous studies investigating aortic aneurysms in this ApoE^{-/-} mouse model (Cao, et al. 2010, Nandlall, et al. 2014) have so far not investigated specific differences in echogenicity and composition of AAA associated thrombi using in vivo high-frequency ultrasound.

The aim of this study was to evaluate the potential of high-frequency-ultrasound for in vivo characterization of AAA associated thrombi in an apolipoprotein E-deficient (ApoE^{-/-}) mouse-model. Histopathology was used as ex vivo reference standard. Magnetic resonance imaging (MRI) was used to validate the in vivo findings regarding the detection of AAA associated thrombi.

Materials and Methods

Animal experiments

All procedures were performed according the guidelines and regulations of the United Kingdom Home Office. Aortic abdominal aneurysms were created in male, homozygous C57BL/6J ApoE knockout mice (8 weeks old) from Charles River Laboratories (Edinburgh, UK) by osmotic mini pump implantation. After intramuscular administration of a combination of Medetomidin (500 μ g/kg), Fentanyl (50 μ g/kg) and Midazolam (5 mg/kg), a minipump (Alzet model 2004, Durect Corp, Cupertino, CA, United States), was implanted subcutaneously in the dorsal region of the neck. Angiotensin II or saline (sham-operated group, n=6) were continuously infused with a rate of 1 μ g/kg/min over a course of 4 weeks. After one, two, three and four weeks of Angiotensin II infusion an ultrasound and MRI imaging session was performed in general anesthesia. At each time point, eight animals were scanned. Following the imaging, mice were euthanized and exsanguinated by arterial perfusion of phosphate buffered saline at a pressure of 100 mm Hg. For histological examinations, an additional perfusion of 10% formalin was performed followed by excision of the aorta including the right renal artery and last pair of intercostal arteries.

In vivo ultrasound imaging

To perform high-frequency ultrasound imaging, animals were anesthetized and placed supine on a heated table. Hair was removed using a depilatory cream. 2D-transverse and sagittal images of the suprarenal abdominal aorta were performed using a Vevo 770 high-resolution ultrasound imaging system (VisualSonics, Toronto, Canada) with 30 MHz transducer (RMV 707B). Cine transversal and sagittal images were acquired by the ECG-based kilohertz visualization (EKV) reconstruction technique that synthesizes images from a series of heart rhythm cycles and

reconstructs one representative heart cycle that is spatially precise and synchronized to the animal's ECG (VisualSonics software, Toronto, Canada).

For in vivo ultrasound imaging a field of view (FOV) of 12.0 x 12.0 mm² was used. The axial spatial resolution was 55 μ m. A lateral spatial resolution of 115 μ m was achieved. For the ECG-based kilohertz visualization (EKV) modus provided by the ultrasound system the signal element transducer worked on a line by line principal. The transducer received and transmitted a pulse repetition frequency of 8000 Hz. A signal on the radiofrequency signal indicated the time of pulse transmission for all imaging data acquisition. The electrocardiogram (ECG) was in all animals derived using electrodes available on the fixation module.

Animal handling and in vivo magnetic resonance imaging

All imaging sessions were performed using a clinical 3 Tesla Achieva MR system (Philips Healthcare, Best, The Netherlands) with a clinical gradient system (30mT/m, 200mT/m/ms) and a microscopy coil (47 mm, Philips healthcare, Best, the Netherlands). Following anesthesia injection, the animals were positioned in a supine position on the microscopy (47 mm in diameter) single loop coil. During the imaging session body temperature (37°C) was monitored using a MR-compatible heating system (Model 1025, SA Instruments Inc, Stony Brook, NY, United States). To localize the abdominal aorta, a low-resolution three-dimensional gradient echo scout scan was performed with the following imaging parameters: Imaging matrix = 160 x 160, field of view = 20 x 20 x 10 mm, slice thickness = 0.5 mm, inplane spatial resolution=0.3 x 0.3 mm (reconstructed 0.13 x 0.13 mm), flip angle = 60 °, repetition time (TR) sequence = 37 ms and echo time (TE) 7.7 ms. Subsequently, for specific visualization of the aorta a 2D TOF angiography (two-dimensional time-of-flight angiography) in transverse orientation was performed using the same parameters.

Imaging parameters of the inversion recovery 3D fast gradient echo MRI scan were field of view = 3 cm, imaging matrix = 300, spatial resolution inplane = 0.1×0.1 , slice thickness 0.5 mm (reconstructed slice thickness = 0.25 mm), 40 slices, repetition time / echo time = 28 / 8.2 ms, flip angle = 30 degrees.

Aortic aneurysm morphometry

Following exsanguination, the suprarenal aorta, including the last pair of intercostal artery branches and the right renal artery were surgically excised. The left renal artery and the last pair of intercostal artery branches were used as landmarks for a coregistration of histology with high-frequency ultrasound and MR imaging. Morphometric measurements were performed on elastin-stained sections using ImageProPlus software (ImageProPlus, MediaCybernetics, Rockville, MD, United States). The percentage Miller's Elastica van Gieson (EvG) stain per adventitial area in the region enclosed by the adventitia and the lumen was assessed. The percentage calculation was performed manually. First the overall area of the Elastica van Giesson stain was assessed and in a second step the overall adventitial area was measured. Then, the percentage calculation was performed based on these values. These techniques have been previously described in (Botnar, et al.) for the characterization of matrix remodeling.

Echogenicity of AAA associated thrombi

For the assessment of the echogenicity of AAA associated thrombi, a "signal thrombus matrix" to "signal blood" ratio was established. Region of interests (ROIs) were drawn to assess the signal of the thrombus as well as the blood. Signal measurements were performed in the transversal and sagittal image orientation at the location of the largest thrombus area. The mean between both

values was calculated. For both ultrasound and magnetic resonance imaging, the drawing of the region of interests (ROIs) was performed using the OsiriX software (version 5.8). All regions of interest in ultrasound and magnetic resonance imaging were manually identified and drawn. In all animals, aortic aneurysms could be clearly distinguished from the surrounding tissues. In ultrasound, the thrombus was detected based on the relative dark signal of the thrombus compared to the relatively bright signal of surrounding tissues. On MRI, the thrombus could be identified based on the typical T1 signal changes of thrombus tissue, compared to the surrounding tissue.

Histological and immunohistochemical analysis of aortic aneurysms

Aortas were perfused with 10% formalin followed by excision of the abdominal aortic aneurysm. For histological sections, aneurysms were embedded in paraffin and cut every 40 μm , starting from the proximal end of the aneurysm. The sections (6 μm) were subsequently stained for Miller's Elastica van Gieson stain and hematoxylin and eosin (HE). For morphology measurements the percentage calculation was performed manually. First the overall area of the Elastica van Giesson stain was assessed and in a second step the overall adventitial area was measured. Then, the percentage calculation was performed based on these values.

Interobserver agreements for high-frequency ultrasound measurements

All images were analyzed independently in a randomized order and blinded to the according other imaging modalities. Area size was recorded for each measurement.

Statistical analysis

Data are expressed as mean \pm standard deviation. To determine continuous variables, a student's

186 t-test (unpaired, 2-tailed) was used. Statistical comparison of groups was performed by analysis of
187 variance (ANOVA) followed by the Bonferroni correction. $P < 0.05$ was used to indicate
188 significance.

Results

Development of abdominal aortic aneurysms and associated thrombi

In the control or sham animals, no development of AAAs was observed (Fig. 1). In the disease group, the continuous infusion of Angiotensin II in ApoE^{-/-} mice led to a reliable development of AAAs in vivo (Fig. 2). Using high-frequency ultrasound, the normal healthy aorta and the aortic dilation at different stages of AAA development could be clearly visualized (Fig. 2). In the control group the abdominal aortic area was the smallest ($1.09 \pm 0.16 \text{ mm}^2$) and no thrombus was detected. In the Angiotensin II group, a steady increase of the size of the aortic lumen was observed from week one to week four ($2.26 \pm 0.92 \text{ mm}^2$ to $3.87 \pm 1.26 \text{ mm}^2$). After one week, an average thrombus area of $2.23 \pm 0.71 \text{ mm}^2$ was measured using high-frequency ultrasound. After two weeks of Angiotensin II infusion an average area of $2.48 \pm 0.81 \text{ mm}^2$ was measured. The increase in thrombus area plateaued after three and four weeks within average area of $2.89 \pm 0.77 \text{ mm}^2$ and $2.86 \pm 0.90 \text{ mm}^2$.

Quantification of thrombus remodeling by high-frequency ultrasound compared to histopathology

Using high-frequency ultrasound, it was not only feasible to detect the development of AAA associated thrombi, but it was also possible to assess relative signal differences in echogenicity of the thrombus matrix at different stages of aneurysm development. Fresh thrombi early after the onset of AAA development (one week) showed a relatively low echogenicity (dark thrombus matrix) with a “signal thrombus matrix” to “signal blood” ratio of 1.61 ± 0.33 (Fig. 2 a2). After two weeks a slight, however, not significant increase of signal to 1.82 ± 0.29 was observed. After three weeks a significant ($p < 0.05$) increase in signal (inhomogeneous bright thrombus matrix,

Fig. 2 a7) with a “signal thrombus matrix“ to “signal blood” ratio to 2.05 ± 0.44 was measured, reflecting a remodeling of the extracellular matrix on histopathology (Fig. 2 a10). After four weeks a further, however, smaller increase of “signal thrombus matrix“ to “signal blood” ratio of 2.14 ± 0.52 was observed, indicating a further remodeling.

On histopathology, the development of an AAA associated thrombus was observed one week after onset of the Angiotensin II infusion. The matrix of the thrombi at such an early stage did not show relevant signs of remodeling (Fig. 2 a4, a5). Only a relatively low percentage ($7.8 \pm 3.5 \%$) of elastic fibers was measured on the Elastica van Giesson stain, resulting mainly from the “normal” elastic lamina in the aortic wall. During week two, a slight increase ($10.4 \pm 1.7 \%$) in expression of elastic fibers was seen on histopathology. On week three, a relatively strong and pronounced remodeling of the thrombus ($13.2 \pm 3.7 \%$) with a strong expression of elastic fibers was observed in the Elastica van Giesson stain. The elastic fibers were expressed throughout the whole matrix of the thrombus. Additionally, a remodeling of the surrounding adventitia with elastic fibers was observed. At week four a slight however not significant additional increase ($14.29 \pm 4.13 \%$) in elastic fiber expression was measured (Fig. 2 a10). A significant difference in “signal thrombus matrix” to “signal blood” ratio was measured between week one and week three ($p < 0.05$) and between week two and week four ($P < 0.05$, Fig. 3a). Additionally, the correlation between the echogenicity of the thrombus (“signal thrombus matrix” to “signal blood” ratio) measured by high-frequency ultrasound and the relative elastin expression (“elastin stain area” to “adventitial area ratio”) measured by ex vivo histology was assessed (Fig. 3b). A close and significant correlation between these in vivo and ex vivo measurements was found ($y = 0.10x + 0.81$, $R^2 = 0.81$, $p < 0.05$).

Detection of AAA associated thrombus and assessment of thrombus size using high-

frequency ultrasound compared to magnetic resonance imaging and histopathology

The AAA associated thrombus size was assessed in vivo using both, high-frequency ultrasound and magnetic resonance imaging in the same animals (Fig. 2). At all stages of disease development, in vivo high-frequency ultrasound enabled a clear visualization of the thrombus, compared to MRI as reference standard. In vivo thrombus size measurements using high-frequency ultrasound showed a close correlation with in vivo magnetic resonance imaging ($y = 0.87x + 0.003$, $R^2 = 0.92$, $p < 0.05$) (Fig. 4). No significant differences ($p < 0.05$) between absolute size measurements existed between high-frequency ultrasound and magnetic resonance imaging.

In vivo high-frequency ultrasound measurements also showed a close correlation with ex vivo size measurements on histopathology using the Elastica van Giesson stain ($y = 1.21x + 0.26$, $R^2 = 0.87$). In vivo area measurements in high-frequency ultrasound were slightly larger compared to area measurements and ex vivo histology, however differences were not significant ($p > 0.05$). The trend towards smaller size measurements on ex vivo histology can be explained by tissue shrinkage, which results from the processing of the tissue samples. In vivo magnetic resonance imaging also showed a close correlation with ex vivo measurements of thrombus size ($y = 1.20x - 0.014$, $R^2 = 0.93$).

Interreader agreement for the assessment of thrombus size using high-frequency ultrasound

The reproducibility of high-frequency ultrasound size measurements was also investigated by two readers. Interobserver correlation for thrombus area measurements in high-frequency ultrasound showed a strong and significant correlation between both readers ($R^2 = 0.86$; $p < 0.05$) (Fig. 5). 95% confidence intervals (CIs) ranged from -0.57 to 0.62. The reproducibility of magnetic resonance measurements was also assessed. A close interobserver correlation ($R^2 = 0.88$; $p < 0.05$)

258 was measured. The associated 95% confidence intervals were small with a range from -0.40 to
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Discussion

This study demonstrated, that high-frequency-ultrasound enables the reliable in vivo quantification of extracellular matrix remodeling at different stages of thrombus development based on the thrombus echogenicity. Late stage highly remodeled thrombi showed a significantly higher echogenicity compared to early/fresh fibrin rich thrombi. As highly remodeled thrombi were shown to stabilize aortic aneurysms, such an in vivo parameter could be useful for the assessment of the AAA associated rupture risk (Saraff, et al. 2003).

Clinical relevance of aortic aneurysms and imaging modalities for the detection of aortic aneurysms

As a consequence of their constantly increasing incidence and their fatal complications in case of rupture, the role of AAAs in the population becomes more and more important (Schmitz-Rixen, et al. 2016). In most cases, AAAs are asymptomatic until rupture occurs. In approximately 75% of patients the rupture of AAA is lethal (Bown, et al. 2002). Currently, it is recommended that patients with aneurysms larger than 5.5 cm and/or with a growth rate of more than 1 cm a year should undergo surgery. In small aneurysms (< 3.5 cm) close follow-up investigations by imaging can be performed. However, there is an ongoing debate regarding the best possible management of medium sized (4 to 5.5 cm) AAAs (Lederle, et al. 2002). Consequently, novel biomarkers are needed to improve assessment of the AAA risk of rupture. Up to date, the only clinically established parameter is the aortic diameter, which is measured in vivo. Different studies demonstrated that this parameter is limited regarding the prognosis of AAA progression and rupture (Lederle, et al. 2002). Additionally, aortic diameter has so far not been validated as a reliable risk factor by population-based studies (Sharp and Collin 2003).

Due to the absence of symptoms during the development of aortic aneurysms, early diagnosis remains challenging. In clinical practice abdominal ultrasound, computed tomography (CT) and magnetic resonance imaging represent the current reference standard for the assessment of AAAs. Each of these imaging modalities was shown to have a high diagnostic sensitivity for the detection of aortic aneurysms in vivo (Thompson, et al. 2002) as well as a high spatial resolution and soft tissue contrast (Buijs, et al. 2013, Forsythe, et al. 2016, Hong, et al. 2010, Sakalihasan, et al. 2005). However, computed tomography and magnetic resonance imaging are relatively cost intensive imaging modalities and CT has the additional disadvantage of using ionizing radiation, which is especially relevant in a young patient collective and in patient collectives which require recent follow-up examinations. By contrast, ultrasound imaging is a reliable, cost effective and radiation free method for the assessment, follow-up surveillance as well as population screening of patients with suspected aortic aneurysms (Buijs, et al. 2013, Sakalihasan, et al. 2005).

Experimental models for the characterization of AAAs

Experimental models play an important role for the investigation of the pathophysiology of AAAs as they enable the simulation of these changes in vivo. Because of their excellent genetic and physiological characterization, the majority of experiments are performed with rodents (Brockmann, et al. 2007). Several different animal models have been developed for studying the pathology of aortic aneurysms (Trollope, et al. 2011). AngII infusion of apolipoprotein E-deficient mice via osmotic minipumps with the associated increase in blood-pressure represents one of the most frequently used models with a reliable and fast development of AAAs (Daugherty, et al. 2000). Another advantage of this model is, that AAAs develop spontaneously and are highly reproducible, while no surgical intervention in the aorta is required.

Ultrasound for the detection and characterization of AAA associated thrombi

The published literature investigating high-frequency ultrasound in the ApoE^{-/-} mouse model with angiotensin II minipumps so far focused on the identification of aneurysms as well as size measurements or staging. Cao et al. (Cao, et al. 2010) studied the fluid mechanics in developing AAAs by ultrasound imaging and examined macrophage infiltration. Complex fluid flow patterns in AAAs were observed and already evident at day 7 post-Ang II infusion with unsteady flow, shed vortices that impinge upon the distal wall, and re-circulating flow which play a critical role in growth and rupture of AAAs. Furthermore, prominent inflammatory macrophage infiltration at the site of elastic media breakdown could be found. Although they identified an area in which local fluid mechanics are favorable to the formation of thrombus, there were no further investigation regarding thrombus composition or remodeling. Another group (Nandlall, et al. 2014) used Pulse Wave Imaging (PWI) to monitor and stage AAA progression. In AAA sacs, wave speeds were significantly lower and exhibit more variability. Additionally, fissured or ruptured AAAs showed changes in the wall displacements induced by pulse waves. An observed reduction in displacement in the aneurysm sacs could be explained by the presence of a thrombus but no further investigation of the thrombus itself were made. This study hypothesized that PWI could be used in future to supply complementary information to that provided by standard B-mode imaging. Martin-McNulty et al. (Martin-McNulty, et al. 2005) and Barisione et al. (Barisione, et al. 2006) were the first to establish high-frequency ultrasound for detection of AAAs in mice. Both studies analyzed the lumen dimensions by two dimensional ultrasound imaging and histologic analysis of the same regions, which shows a close agreement. In contrast to these studies, this study investigated specific differences in echogenicity and composition of AAA associated thrombi.

The majority of AAAs is associated with a thrombus that adheres to the aneurysmal wall (Hans,

et al. 2005, Kazi, et al. 2005, Labruto, et al. 2011, Vorp, et al. 2001). Clinical studies have shown that transabdominal ultrasound enables the detection of AAA associated thrombi (Wanhainen, et al. 2002). However only a limited number of clinical studies have specifically investigated the composition of thrombi in vivo using ultrasound. In general, it was shown that thrombi are relatively echogenic compared to the echo free aortic lumen. Additionally, it was demonstrated that ultrasound enables the detection of fissures and dissections in AAA associated thrombi (Wanhainen, et al. 2002). To the best of our knowledge, specific differences in echogenicity of AAA associated thrombi has not been specifically evaluated yet. However, in the context of atherosclerosis, factors which lead to a change of echogenicity have been investigated. Based on histological analysis it was suggested that echolucent plaques are rich in hemorrhage/fibrin and lipid (Gronholdt, et al. 2001, Mathiesen, et al. 2001, Tegos, et al. 2001). In contrast, echogenic plaques were shown to be rich in fibrous tissue, which is mainly composed of extracellular matrix proteins, including elastin and collagen (Gronholdt, et al. 1997, Kardoulas, et al. 1996). These observations are in line with our current study, in which we evaluated the potential of high-frequency ultrasound for the detection and characterization of AAA associated thrombi. Using high-frequency ultrasound, it was possible to evaluate the structure of the thrombus matrix and therefore to assess its state of remodeling. As described for atherosclerotic plaques, late stage AAA associated thrombi, which are rich in extracellular matrix proteins (e.g. elastin), show a higher echogenicity compared to early/fresh thrombi which are rich in fibrin/hemorrhage. A close correlation between echogenicity, measured by in vivo ultrasound, and extracellular matrix remodeling, measured in ex vivo histology, was found. In addition, a high interobserver reproducibility was found for ultrasound as well as MRI measurements. Since late stage highly remodeled thrombi were shown to stabilize aneurysms (Adolph, et al.

1997, Li, et al. 2008, Saraff, et al. 2003, Thubrikar, et al. 2003, Wang, et al. 2002), this imaging approach may represent a new diagnostic tool to improve the risk stratification of patients and to guide therapeutic interventions.

Limitations

One limitation of the used experimental model might be that the location of AAA varies between humans and mice. While AAAs in mice develop in the suprarenal aorta, human AAAs develop in the infrarenal part of the aorta. It is assumed that differences in aortic wall composition and hemodynamics might contribute to this difference.

Conclusion

This study demonstrated, that high-frequency-ultrasound enables the reliable in vivo quantification of extracellular matrix remodeling at different stages of thrombus development based on the thrombus echogenicity.

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Figure Captions List

Figure 1: In vivo imaging of the abdominal aorta in sham operated apolipoprotein E-deficient (ApoE ^{-/-}) mice

After 4 weeks of continuous saline infusion via subcutaneously implanted osmotic minipumps (n=6), ultrasound (**a1-2, a6**) and magnetic resonance (MR) (**a3, a7**) imaging were performed. Longitudinal and corresponding transverse ultrasound images (**a1-2, a6**) as well as transverse MR images (**a3, a7**) show no dilatation or alterations of the aortic wall. The red and orange lines (**a1**) indicate the location of the corresponding transverse images. Histological sections (**a4-5, a8-9**) showed an intact vessel wall without dilatation. EvG indicates Elastica van Gieson staining; HE, hematoxylin and eosin staining. Ao: Aorta.

Figure 2: Development and remodeling of abdominal aortic aneurysm (AAA) associated thrombi in apolipoprotein E-deficient (ApoE ^{-/-}) mice

Longitudinal and corresponding transverse ultrasound images of an ApoE ^{-/-} mouse after one week (**a1,2**) and four weeks (**a6,7**) of Angiotensin II (AngII) infusion (n=8 per group). The formation of an AAA associated intramural thrombus was observed. The perimeter of the thrombus is outlined by the orange and red dotted line (**a2-3, 7-8**). Transverse magnetic resonance images (**a3, a8**) of the same segment show similar characteristics. Histological sections confirmed rupture of the elastic laminae and fibrin rich thrombus formation (**a4-5**, including magnification). After four weeks of AngII infusion, a strong remodeling of the thrombus matrix was observed (**a9-10**). The magnification demonstrates a high expression of extracellular matrix proteins, including elastic

fibers. EvG indicates Elastica van Gieson staining; HE, hematoxylin and eosin staining. Ao: Aorta, Th: Thrombus.

Figure 3: In vivo assessment of thrombus echogenicity in correlation to thrombus remodeling

a: Weekly in vivo ultrasound imaging (n=8 per group) of the thrombus matrix over the time course of Angiotensin II (AngII) infusion resulted in the detection of a significant and constantly increasing echogenicity (signal thrombus to signal blood ratio) of the thrombus matrix. In sham operated mice no thrombus formation was observed. **b:** A significant correlation ($R^2 = 0.81$) between the echogenicity of the thrombus measured by high-frequency ultrasound and the relative elastin expression (“elastin stain area” to “adventitial area ratio”) measured by ex vivo histology was observed. Values are expressed as mean \pm SD.

Figure 4: Comparison of in vivo and ex vivo measurements of thrombus area in abdominal aortic aneurysm (AAA)

To further validate in vivo high-frequency ultrasound measurements, thrombi areas were assessed at the same location by in vivo ultrasound and ex vivo histology. A significant correlations ($p < 0.05$) between in vivo ultrasound (**a**) and ex vivo histology were observed ($R^2 = 0.87$).

Figure 5: Inter-observer variability for ultrasound measurements

520 **a, b:** Inter-observer variability for in vivo high-frequency ultrasound measurements showed a close
521 and significant correlation between both readers ($R^2 = 0.86$, $p < 0.05$) with a relatively small 95%
522 confidence interval ranging from -0.57 to 0.62 in Bland-Altman plots.

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